Hypothesis

# Prokaryotic origins for the mitochondrial alternative oxidase and plastid terminal oxidase nuclear genes

Patrick M. Finnegan[a,*], Ann L. Umbach[c], Jackie A. Wilce[b]

[a] *Plant Molecular Biology Group and School of Plant Biology, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia*
[b] *School of Biomedical and Chemical Sciences, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia*
[c] *DCMB Group, Biology Department, Box 91000, Duke University, Durham, NC 27708-1000, USA*

Received 13 August 2003; revised 28 September 2003; accepted 28 September 2003

First published online 18 November 2003

Edited by Horst Feldmann

**Abstract The mitochondrial alternative oxidase is a diiron carboxylate quinol oxidase (Dox) found in plants and some fungi and protists, but not animals. The plastid terminal oxidase is distantly related to alternative oxidase and is most likely also a Dox protein. Database searches revealed that the α-proteobacterium *Novosphingobium aromaticivorans* and the cyanobacteria *Nostoc* sp. *PCC7120*, *Synechococcus* sp. *WH8102* and *Prochlorococcus marinus* subsp. *pastoris CCMP1378* each possess a Dox homolog. Each prokaryotic protein conforms to the current structural models of the Dox active site and phylogenetic analyses suggest that the eukaryotic Dox genes arose from an ancestral prokaryotic gene.**


*Key words:* Alternative oxidase; Plastid terminal oxidase; IMMUTANS; Molecular phylogeny; Cyanobacteria; Proteobacteria; Diiron carboxylate proteins

## 1. Introduction

Plant mitochondria are distinct from their animal counterparts by possessing a cyanide-resistant, salicylhydroxamic acid-sensitive terminal oxidase within the inner membrane that is additional to the well-known cyanide-sensitive cytochrome *c* oxidase. The alternative oxidase (Aox) directly transfers electrons from ubiquinol to molecular oxygen and has been found in all land plants examined, many, but not all, fungi and some protists. Aox is of interest because it is not energy conserving and uncouples electron transport from ATP synthesis. The ability of at least the plant enzyme to compete directly with respiratory complex III for electrons within the ubiquinone pool [1,2] means that Aox has the capacity to dramatically alter the respiratory efficiency of oxidative phosphorylation.

The ability of Aox to catalyze the four electron reduction of oxygen to water suggests the presence of a coupled transition metal center at its active site [3]. Early spectroscopic examinations were inconclusive in identifying a metal center (reviewed in [4]). However, indirect studies suggested Aox from *Pichia anomala* (previously named *Hansenula anomala*) [5] and *Trypanosoma brucei* [6] require $Fe^{2+}$ for catalytic function.

Nuclear-derived Aox cDNAs or genes have been isolated from numerous plants, fungi and protists. Noting spectroscopic similarities between Aox and the oxo-bridged diiron carboxylate protein subunits of methane monooxygenase and ribonucleotide reductase, and the presence of specific motifs in all of these proteins, Siedow et al. [3] modeled the Aox active site after the solved crystal structure of the three diiron proteins that were available. The original model has been extensively revised, using the diiron $\Delta^9$ desaturase (pdb:1AFR) as a template, to take into account more recent sequence information and evolutionary considerations [7,8]. Although the Aox sequence shows essentially no homology with other diiron carboxylate proteins, apart from motif placement, its modeled active site structure conforms to the characteristic diiron carboxylate protein four-helical bundle that provides ligands for binding the diiron center [7]. An electron paramagnetic resonance (EPR) signal that is consistent with the presence of an oxo-bridged diiron center has recently been detected in an *Arabidopsis thaliana* Aox [9] providing direct evidence that Aox is a diiron carboxylate protein.

The cloning of the *Im* gene from *Arabidopsis* [10,11] has strengthened and extended our understanding of Aox active site structure [8]. The *Im* gene encodes a plastid-localized terminal oxidase (Ptox [12]), with over-all sequence similarity to Aox [10,11]. In the greening plastid, Ptox is important for carotenoid synthesis. When expressed in *Escherichia coli*, Ptox confers cyanide-resistant, propyl gallate-sensitive oxygen consumption to membrane fractions [12], demonstrating that this enzyme is not only similar to Aox in sequence, but also in the reaction it catalyzes and presumably in the structure of its active site. The similarities in catalysis, inhibitor response and amino-acid sequence [10–12] between Aox and Ptox strongly suggest that Ptox, like Aox, is a diiron carboxylate quinol oxidase (Dox).

The evolutionary origins of the eukaryotic Dox proteins are

*Corresponding author. Fax: (61)-8-9380 1001.
*E-mail addresses:* pfinnega@agric.uwa.edu.au (P.M. Finnegan), umbacha@acpub.duke.edu (A.L. Umbach), jwilce@chem.uwa.edu.au (J.A. Wilce).

*Abbreviations:* Aox, alternative oxidase; Ptox, plastid terminal oxidase; Dox, diiron carboxylate quinol oxidase; EPR, electron paramagnetic resonance; *No*Dox, *Nostoc* sp. *PCC7120* Dox; *Pm*Dox, *Prochlorococcus marinus* subsp. *pastoris CCMP1378* Dox; *Na*Dox, *Novosphingobium aromaticivorans* Dox; *Sy*Dox, *Synechococcus* sp. *WH8102* Dox

0014-5793/03/$22.00 
doi:10.1016/S0014-5793(03)01309-7

A

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AtAOX1a MMITRGGAKAAKSLLVAAGPRLFSTVRTVSSHEALSASHILKPGVTSAWIWTRAPTIGGMRFASTITLGE  70
NaDox   ------------------------------------------------------------------------  -
AtPtox  ----------------------------------------------MAAISGISSGTLTISRPLVTLRR  49
NoDox   ------------------------------------------------------------------------  -
PmDox   ------------------------------------------------------------------------  -
SyDox   ------------------------------------------------------------------------  -

AtAOX1a KTPMKEEDANQKKTENESTGGDAAGGNNKGDKGIASYWGVEPNKITKEDGSEWKWNCFRPWETYKADITI 140
NaDox   ------------------------------------------------------------MIPPFI   6
AtPtox  SRAAVSYSSSHRLLHHLPLSSRRLLLRNNHRVQATILQDDEEKVVVEESFKAETSTGTEPLEEPNMSSSS  93
NoDox   ------------------------------------------------------------------  -
PmDox   ------------------------------------------------------------------  -
SyDox   ------------------------------------------------------------------  -

                                    1
AtAOX1a DLKKHHVPTTFLDRIAYWTVKSLRWPTDLFFQRRYGCRAMMLETVAAVPGMVGGMLLHCKSLRRFEQSG- 209
NaDox   DLSVHHKPGGLSDRIAFGFTKALRWCADTFFAERYGHRAVVLETVAAVPGMVGATINHLACLRRMCDDK-  75
AtPtox  TSAFETWIIKLEQGVNVFLTDSVIKILDTLYRDRTYARFFVLETIARVPYFAFMSVLHMYETFGWWRRA- 162
NoDox   --------------MIRLLVGILVFVINTVYRDRPYPRFYVLETVARVPYFSYLSVLHLYETLGWWRKA-  55
PmDox   -----------MKNLNSFILNNTVKILDFVYSDRHLQRFWVLEVIARSPYFAFLSVLHFKESLGLKNDIT  59
SyDox   -----METKTRKAATAHKIIEAGEGVLNLVWGKADERRAASLEIIARTAYTAEESACHYLETIGLDKEGT  65

          2                                   3
AtAOX1a --GWIKALLEEAENERMHLMTFM-EVA-KPKWYERALVITVQGVFFNAYFLGYLISPKFAHRMVGYLEEE 275
NaDox   --GWIKTLMDEAENERMHLMTFI-EIS-KPTLFERAVIMGVQWVFYLFFFGLYLVSPKTAHRVVGYFEEE 141
AtPtox  --DYLKVHFAESWNEMHHLLIME-ELGGNSWWFDRFLAQHIATFYYFMTVFLYILSPRMAYHFSECVESH 229
NoDox   --DWLKVHFAESWNELHHLLIME-SLGGAGFWGDRFLAKTAALIYYWIIIAVYFVSPHSAYNFMEQVEQH 122
PmDox   MFL-MKEHFYQAINETEHLKEME-KRGGDKFWIDRFLARHLVLVYYWIMVFYYFCSPRNAYDVNIKIEEH 127
SyDox   IRDTLELARYQDTNEQTHEDIFARDLDGLKNWGDRFLARHIAVVIYWVFAITTLIDHEMAALLGEAVEVE 135

                                    4
AtAOX1a AIHSYTEFLKELDKGNIENVPAPAIAIDYWR-------------------LPADATLRDVVMVVRADEAH 326
NaDox   AVISYTHYLAEIDQGRSANVPAPAIAKRYWG-------------------LPDNAMLRDVVLVVRADEAH 192
AtPtox  AYETYDKFLKASGEE-LKNMPAPDIAVKYYTGGDLYLFDEFQTSRTPNTRRPVIENLYDVFVNIRDDEAE 298
NoDox   AYSSYDKFLTTHEAE-LKTQPAPEVAKTYYRDGDLYMFDEFQTAHSPSERRPNIDNLYDVFVAIRDDEME 191
PmDox   AFNTYTKYLKDHPE-------------------------------------------DQKIKEIAQDELN 154
SyDox   AVKTYRRMLKEQPEE-WLDQPAAPTATHYWE---------KPNSMWRVRGDHMPGSMRDVVEAIVRDEAD 195

AtAOX1a HRDVNHFASDIHYQGRELKEAPAPIGYH-------------------------- 354
NaDox   HRDVNHGFANELAGLPVAEPAACPPHHALEPNWKKAA----------------- 229
AtPtox  HCKTMRACQTLGSLRSPHSILEDDDTEEESGCVVPEEAHCEGIVDCLKKSITS 351
NoDox   HVKTMVACQQTNAKYSLKSPHNPEAKTVVLTNAQMNTQV-------------- 230
PmDox   HVEELNEALAMITQS------------------------------------- 169
SyDox   HVKANSKKATAF--------------------------------------- 207
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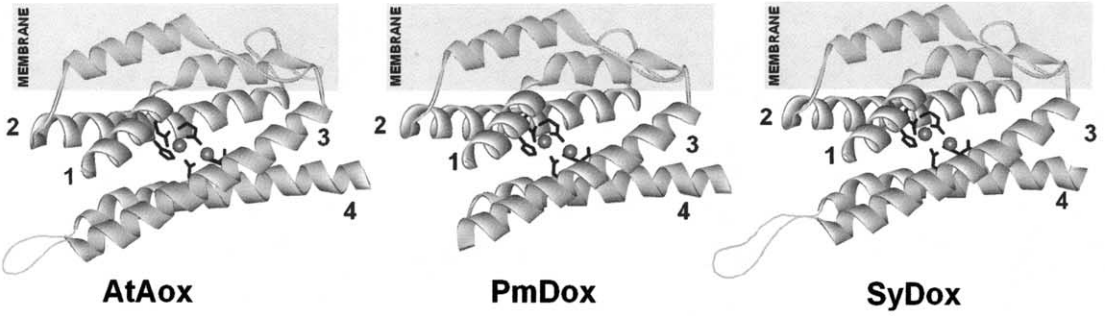
B



unknown. The preponderance of cellular, biochemical and molecular evidence supporting a prokaryotic origin for mitochondria and plastids points to the α-proteobacteria and cyanobacteria as being the closest living relatives to the ancestral mitochondrion and plastid, respectively [13]. However, Aox activity among the prokaryotes has never been satisfactorily demonstrated and gene homologs for *Aox* or *Im* have not been found among the prokaryotes, leaving open the question of the origin of the Dox gene family. The progress of genome sequencing projects has provided new opportunities to answer this question. Database searches now indicate that the Dox gene family has its origins in the prokaryotic world and suggest that the divergence of Aox and Ptox genes pre-dates the origin of the mitochondrial and plastid progenitor species.

## 2. Results and discussion

Recent database searches revealed predicted proteins amongst the entries of four prokaryote genome sequencing projects with striking sequence similarity to the *Arabidopsis* Dox proteins, Aox (*At*Aox) and Ptox (*At*Ptox). A protein from the α-proteobacterium *Novosphingobium aromaticivorans* (*Na*Dox, accession ZP_00095227) genome project had 56% sequence identity with *At*Aox1a and proteins from three cyanobacteria genome projects had 51, 42 and 26% sequence identity with *At*Ptox (Fig. 1A). The Ptox-like proteins were found in the *Nostoc* sp. *PCC7120* (*No*Dox, accession NP_486136), *Prochlorococcus marinus* subsp. *pastoris CCMP1378* (*Pm*Dox, accession NP_892455) and *Synechococcus* sp. *WH8102* (*Sy*Dox, accession NP_896980) projects, respectively.

The newly identified prokaryotic Dox-like sequences were consistent with the proposed structural features of the eukaryotic Dox protein family, as modeled on the diiron carboxylate proteins [7,8]. Aox is currently viewed as a diiron carboxylate protein embedded in the inner face of the mitochondrial inner membrane [7] (see Fig. 1B). The active site comprises two pairs of antiparallel helices. A single glutamate residue in the more N-terminal helix of each helix pair (helices 1 and 3) and the glutamate and histidine residues in EXXH motifs located in the more C-terminal helix of each helix pair (helices 2 and 4) are juxtaposed to coordinate two iron atoms at the catalytic center. Alignment of the prokaryotic Dox-like sequences with the eukaryotic Dox sequences showed conservation across the entire family of the four-helix bundle central to the proposed active site structure, and, most importantly, strict conservation of the six residues put forward as iron ligands (Fig. 1A). The overall structural conservation was confirmed by these protein sequences mapping appropriately to the four-helical bundle of the diiron $\Delta^9$ desaturase crystal structure (Fig. 1B).

Accumulating evidence from site-directed mutagenesis studies on Aox isoforms supports the importance of the putative iron ligating residues. Changing the glutamate in the EXXH motif of helix 2 abolished the characteristic EPR signal of an *At*Aox [9], while changing the histidine or glutamate residues in this motif or in the EXXH motif of helix 4 inactivated trypanosome and/or *Sauromatum guttatum* Aox [6,14]. The importance to enzyme activity of the strictly conserved glutamate residue in helix 1 has not been experimentally tested.

The identity of the iron ligating glutamic acid residue in helix 3 has been uncertain because of the presence of three consecutive glutamate residues in the eukaryotic enzymes. Changes to the N-terminal residue of the triplet in *At*Aox1a eliminated the distinctive EPR signal [9], while changes to the second or third residues in the trypanosome enzyme [6,15] or the third residue in the *S. guttatum* enzyme [14,16] abolished activity. These results may indicate that there is species specificity in the employment of these residues in iron binding, or perhaps the ability of the native ligand to interact with iron is disrupted by particular changes at the other two positions. The latter possibility is supported by the finding that the *Dictyostelium discoideum* Aox (accession BAB82989) and *Prochlorococcus* Dox-like proteins lack the C-terminal glutamate of the triplet, while the plant Ptox, and *Nostoc* and *Synechococcus* Dox-like proteins lack all but the N-terminal glutamate residue (Fig. 1A). The prokaryotic sequences, then, support the suggestion that the more N-terminal glutamate residue of the triplet is the natural iron ligand [8].

A fifth helical region lying between helices 2 and 3 of the four helix bundle in eukaryotic Dox proteins [7,8] was also found in the prokaryotic Dox-like sequences. Kyte–Doolittle hydropathy analyses showed that this helical region and the C-terminal half of helix 1 contained the most hydrophobic domains of all Dox proteins [7], including the newly identified prokaryotic proteins (data not shown). The position of the hydrophobic helical regions alongside one another at one surface of the four-helix bundle has led to the proposal that these domains are responsible for the membrane-association of the Dox proteins [7] (see Fig. 1B). Alternatively, the hydrophobic domains may be involved in protein–protein interactions [7], including those responsible for dimerization of land plant Aox subunits [17].

Of the cyanobacterial Dox-like proteins, *No*Dox had the

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Fig. 1. A: Alignment of Dox protein sequences. Deduced protein sequences with similarity to *A. thaliana* Aox proteins were identified using the BLAST algorithm available at the National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov) to interrogate the non-redundant protein sequence database. Extracted Dox sequences from *Arabidopsis* (*At*Aox1a, accession BAA22625 and *At*Ptox, accession CAA06190), *Novosphingobium aromaticivorans* (*Na*Dox, accession ZP_00095227), *Synechococcus* sp. *WH8102* (*Sy*Dox, accession NP_896980), *Prochlorococcus marinus* subsp. *pastoris CCMP1378* (*Pm*Dox, accession NP_892455) and *Nostoc* sp. *PCC7120* (*No*Dox, accession NP_486136) were aligned using ClustalX v1.81 [23]. Residues that are identical (black background) or similar (gray background) in more than half of the sequences in the figure are highlighted. The four helical regions (bars) defined by Andersson and Nordlund [7] are shown above the sequences. The strictly conserved iron-ligating glutamate and histidine residues are indicated (arrowheads). The conserved central region of the alignment used to construct Fig. 2 is bordered by arrows below the sequences. Numerals on the right indicate amino-acid residues in the corresponding sequence. B: Schematic representations of the *At*Aox, *Pm*Dox and *Sy*Aox active site structures as proposed by Andersson and Nordlund [7] and based on the diiron $\Delta^9$ desaturase crystal structure (pdb: 1AFR). The numerals indicate the four helices (ribbons) involved in iron binding. The sequences between helices 3 and 4 do not align with the $\Delta^9$ desaturase sequence, are variable in length and are shown as unmodeled loops for *At*Aox and *Sy*Dox. This segment is absent from the *Pm*Dox sequence. Iron atoms (spheres) are shown coordinated by the proposed active site glutamic acid and histidine residues (lines). The protein molecules are partially embedded in a membrane (shaded) via a fifth helix (located between helices 2 and 3) and the C-terminal half of helix 1.

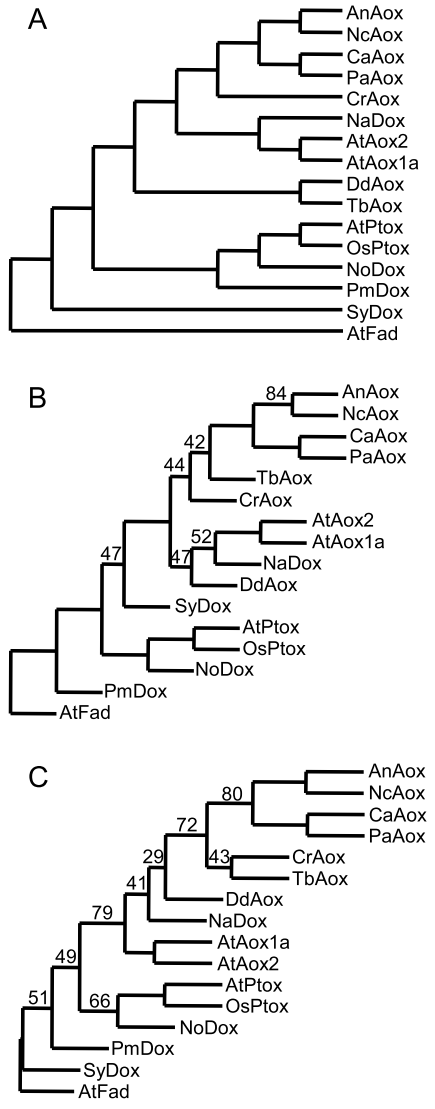


Fig. 2. Phylogenetic analysis of Dox sequences. The central conserved region of the Dox sequences aligned in Fig. 1A, corresponding to residues 178 to 327 of *Arabidopsis* AtAox1a, from the species indicated in the legend to Fig. 1A and from *Aspergillus nidulans* (AnAox, AAN39883), *Neurospora crassa* (NcAox, accession Q01355), *Candida albicans* (CaAox, accession AAF21993), *Dictyostelium discoideum* (DdAox, accession BAB82989), *P. anomala* (PaAox, accession S17517), *T. brucei* (TbAox, accession Q26710), *Chlamydomonas reinhardtii* (CrAox, accession T07947), *A. thaliana* (AtAox2, accession NP_201226) and *Oryza sativa* (OsPtox, accession AAC35554) were aligned using Clustal X. The *Arabidopsis* fatty acid $\Delta^9$ desaturase sequence (AtFad, accession NP_197127.1) was included as an outgroup. Maximum likelihood, parsimony and distance matrix trees were constructed using the PHYLIP [24] algorithms available through the Biomanager web site (biomanager.angis.org.au). A: maximum likelihood tree that arose in 40 of 50 trees built. B: consensus parsimony and C: consensus distance (neighbor joining) trees built from 1000 bootstrap replicates. Bootstrap values $<90\%$ are shown on the relevant branches where branch length is proportional to the bootstrap value.

highest similarity to *At*Ptox (Figs. 1A and 2). The *Sy*Dox and *Pm*Dox sequences align less well with *At*Ptox, with nine and 42 residue deletions relative to *At*Ptox, respectively, occurring between helices 3 and 4. In the case of *Sy*Dox, the nine residue deletion would not impinge on the integrity of helices 3 and 4 of the four-helix bundle, as the interhelical loop is still longer than that in the mitochondrial Aox proteins. In contrast, the deletion in *Pm*Dox results in a substantial shortening of the distance between the iron ligating residues in helices 3 and 4 (Fig. 1), bringing these key amino acids 24 residues closer together than they are in mitochondrial Aox proteins. It is still plausible, however, that the remaining 26 amino acids between the iron ligating residues allow *Pm*Dox to conform to the four-helix bundle of the diiron binding structural motif with shortened helices 3 and 4 (Fig. 1B), but experimentation is needed to confirm this.

Several other key amino acids were conserved throughout the Dox family, supporting the proposed relationship between the prokaryotic and eukaryotic proteins. Residues thought to be important for stabilizing the ligating histidines through hydrogen bonding, N221 and D323 (all numbering corresponds to *At*Aox1a, Fig. 1A), were strictly conserved throughout the family. Residues Y258 and Y280 have been suggested to be important to the catalytic mechanism of Aox, possibly by acting as long-lived radicals at the diiron site [8]. It has been demonstrated that Y280 is essential for *S. guttatum* Aox1 activity [16]. Both Y258 and Y280 were strictly conserved throughout the Dox family, with the exception of Y258 in *Sy*Dox. However, in the latter enzyme, Y251 may be in a position to substitute for Y258 because the aromatic side chains of both residues would be on the same helical face, although exactly two helical turns removed. In all Dox proteins, the hydrophobic pocket surrounding Y280 [8], which contains strictly conserved L182 and A276, along with hydrophobic residues at positions equivalent to A179, F229 and V320, were conserved. Several positively charged residues (R178, H198, R/K240) near the hydrophobic helices and proposed to interact with the phosphate groups in the membrane [8] were also strictly conserved among the Dox proteins.

All of the prokaryotic proteins were shorter than their eukaryotic counterparts, mostly due to N-terminal truncations in the prokaryotic proteins prior to helix 1 (Fig. 1A). The lack of N-terminal extensions in the prokaryotic proteins may be due to differences in the functional requirements of these enzymes. For example, nuclear-encoded eukaryotic Dox isoforms require N-terminal targeting signals to be directed to and imported into the appropriate organelle. Sequences targeting the prokaryotic proteins to the cell membrane are probably unnecessary, as illustrated by the ability of an N-terminally truncated form of *At*Aox1a to insert into and form a functional enzyme in *E. coli* membranes [18]. In this truncated version of *At*Aox1a, only 12 of the original 62 amino acids of the targeting sequence remained in the primary translation product. However, in the truncated *At*Aox1a and other mature land plant and fungal Aox proteins, there are still close to 100 amino acids upstream of the beginning of the first diiron binding helix. For the plant Aox, this N-terminal region functions in the regulation of enzymatic activity through the highly conserved residue C127 [19–21]. The prokaryotic enzymes are likely to lack the regulatory functions as they lacked the region containing C127.

The finding of homologs for nuclear-encoded Dox proteins among the prokaryotes raises the intriguing possibility that Aox and Ptox were acquired by eukaryotes as a by-product of the endosymbiotic events that gave rise to mitochondria and plastids. The phylogenetic relationships among the Dox protein family were examined using maximum likelihood, parsimony and distance methods. The analysis focused on the

most highly conserved region of the Dox sequences (corresponding to residues 178–327 in *At*Aox1a, Fig. 1A), and was restricted by omitting all but representative land plant and fungal Aox and land plant Ptox sequences. The *Arabidopsis* fatty-acid $\Delta^9$ desaturase sequence was included as a distantly related outgroup. Each method produced a topologically similar tree, with the cyanobacterial Dox-like sequences grouping with the plastid Ptox sequences and the α-proteobacterial Dox-like sequence grouping with the mitochondrial Aox sequences (Fig. 2). These strong phylogenetic affinities, coupled with the compelling cellular, biochemical and molecular evidence supporting a cyanobacterial origin for plastids and an α-proteobacterial origin for mitochondria [13], strongly suggest that the Dox protein family arose in an ancestral prokaryote, radiated into both the cyanobacterial and α-proteobacterial lineages and then on into the mitochondrial and plastid lineages, respectively, as a result of the endosymbiotic events that gave rise to the respective organelles.

All the trees generated from this small set of prokaryotic sequences agreed that the cyanobacterial Dox-like sequences were more deeply-rooted than the α-proteobacterial Dox-like sequence (Fig. 2), suggesting that the latter was derived from the former. Among the cyanobacterial sequences, *No*Dox always grouped closely to the land plant Ptox sequences, supporting a monophyletic origin of the land plant Ptox sequences from the cyanobacterial ancestor of modern plastids. The branching order of *Sy*Dox and *Pm*Dox was not clear from this analysis. The maximum likelihood and distance trees indicated that *Sy*Dox branched first from the Dox lineage, while parsimony analysis placed this sequence either at the root of the mitochondrial Aox branch (47% of trees) or at the root of the *No*Dox/plastid Ptox branch (43% of trees). Bootstrap analysis suggested that the parsimony tree (Fig. 2B) may be a somewhat better reflection of cyanobacterial Dox evolution than the distance tree (Fig. 2C). Despite the uncertainty in the exact branching among the cyanobacterial sequences, it is clear that cyanobacterial Dox-like enzymes are ancestral to both mitochondrial and plastid Dox enzymes.

All the tree building methods placed the α-proteobacterial *Na*Aox sequence in amongst the mitochondrial sequences, topologically separated from the cyanobacterial sequences. However, there was method-dependent variation in tree topology within the mitochondrial Aox branch. The maximum likelihood tree for this branch (Fig. 2A) was supported by being topologically most similar to trees derived using α- and β-tubulin, actin and elongation factor 1α sequences (see [22]). In the latter trees, the trypanosome sequences were deeply rooted, with the fungal and land plant sequences diverging to form the crown. The similarity between the maximum likelihood tree for Aox and trees built for other protein sequences does not preclude other evolutionary scenarios for mitochondrial Aox proteins.

There are numerous possible explanations for the topological separation of the α-proteobacterial and cyanobacterial Dox-like sequences. For example, the α-proteobacterial and cyanobacterial sequences may have diverged greatly since their last common ancestor in the prokaryotic world. There may also have been convergent, rather than divergent, evolution of some mitochondrial Aox and α-proteobacterial Dox-like sequences since the divergence of these lineages, perhaps due to evolutionary pressures and/or saturation of mutable sites. Alternative scenarios, such as a higher evolutionary rate for *Na*Dox than for mitochondrial Aox sequences, or horizontal acquisition of Aox by the α-proteobacterium are also possible. A trivial explanation for the observed branching is that the Aox sequence attributed to *N. aromaticivorans* is a contaminant of the sequencing library. However, comparisons of the GC content, GC distribution among codons and codon usage between *Na*Dox and other *N. aromaticivorans* genes indicates this is unlikely (data not shown). The true evolutionary history of the mitochondrial Aox sequences will only be revealed through the analysis of more eukaryotic and prokaryotic Dox sequences.

The apparent prokaryotic origin for Dox genes, followed by radiation of the genes through the eukaryotic lineages as a consequence of mitochondrial and plastid acquisition provides an inclusive explanation for the widespread distribution of extremely well-conserved mitochondrial Aox sequences in organisms as diverse as the trypanosomes, land plants, *Chlamydomonas*, *Dictyostelium* and fungi. A prokaryotic origin for Dox would also suggest that the phylogenetically sporadic distribution of Aox among the eukaryotes is most likely due to the loss of the gene in those lineages that lack the enzyme, including animals. Sporadic loss of Aox is supported by the presence of characteristic Aox genes in the yeasts *P. anomala* and *Candida albicans* [25,26], but the absence of an Aox gene in the yeast *Saccharomyces cerevisiae*. Characteristic Aox genes are also found in the kinetoplastid parasites *T. brucei* and *Phytomonas* sp., but are apparently missing from the closely related kinetoplastid parasites *Leishmania* spp. [27]. Finally, an Aox gene is present in the green alga *Chlamydomonas reinhardtii*, but apparently not in its colorless relative *Polytomella* sp. [28]. Therefore, the often-cited lack of Aox in the animal lineage could well be the result of gene loss from an ancestral animal. However, whether all animal lineages lack Aox is uncertain, as very few animals have been examined in sufficient detail and there are intriguing indications that some primitive animals, such as worms, possess cyanide-resistant, salicylhydroxamic acid-sensitive respiration reminiscent of Aox [29,30]. It remains to be determined whether Ptox is as sporadically distributed as Aox. So far, there has not been a well substantiated case for the lack of Ptox in a photosynthetic eukaryote.

The cause of the irregular distribution of Aox among taxa is unclear. There is wide experimental support that Aox provides metabolic flexibility to plants and fungi. The enzyme provides the capacity for non-fermentative recycling of reduced pyrimidine nucleotides and continued flow of carbon through the TCA cycle under conditions where electron flow through the main cytochrome pathway is constricted (e.g. during phosphate limitation or when ATP levels are high) [31]. This outcome is taken to an extreme in *T. brucei*. The blood-borne stage of the parasite lacks cytochrome pathway activity and Aox provides the main path to oxygen for electrons derived from metabolic reactions [27]. Another example of divergent metabolisms is found among yeasts. Those having alternative pathway activity do not have the ability to ferment sugars aerobically. Conversely, those able to ferment aerobically have no alternative pathway activity [32]. Either aerobic fermentation or the alternative pathway accomplishes the same goal of processing respiratory input that is in excess of the cytochrome pathway capacity [32]. There is also strong support for the view that Aox in plants and fungi attenuates the formation of harmful reactive oxygen species by maintain-

ing Q pool reduction levels within acceptable limits [31]. Organisms lacking Aox either do not require the protective or metabolic flexibility afforded by the oxidase or have other enzymes or pathways that provide that flexibility.

It has been hypothesized that the diiron carboxylate protein family originated among anaerobic bacteria in an anoxic world [33]. It has been further proposed that a primitive family member evolved into an oxygen reductase able to scavenge dioxygen during the transition from an anoxic to an oxic atmosphere, thus protecting the host organism from reactive oxygen species generated by the reaction of dioxygen with metal ions, metabolites and cellular macromolecules. The activities of modern Aox and Ptox enzymes, then, are reminiscent of this ancestral enzyme. The finding of prokaryotic Dox genes now establishes a missing link between the eukaryotic Aox and Ptox genes and the prokaryotic diiron carboxylate protein genes, strengthening the phylogenetic relationships that have been drawn from structural alignments [33]. Gomes et al. [33] suggested that a prokaryotic rubrerythrin present in an endosymbiont might have been ancestral to eukaryotic Dox proteins, but the new sequence data analyzed here show that prokaryotic Dox proteins, as a family, were most likely already present before the endosymbiotic events that gave rise to chloroplasts and mitochondria occurred. While it is not yet known whether the prokaryotic Dox enzymes are oxygen reductases, the identification of their genes opens a potentially profitable research area that may provide a greater understanding of the evolution of diiron carboxylate proteins and the role of Dox enzymes in cellular metabolism.

*Acknowledgements:* We thank D.A. Day, J. Whelan and A.H. Millar for helpful discussions and critical reading of the manuscript. P.M.F. acknowledges the support of the Australian Research Council (DP0343849) and A.L.U.'s work is funded by NSF Grant number MCB-0091080.

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